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New Mechanism for Methicillin Resistance in *Staphylococcus aureus*: Clinical Isolates That Lack the PBP 2a Gene and Contain Normal Penicillin-Binding Proteins with Modified Penicillin-Binding Capacity

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Seventeen clinical isolates of *Staphylococcus aureus* (from the United States and Europe) selected for low (borderline)-level methicillin resistance (MIC of methicillin, 2 to 4 μg/ml; MIC of oxacillin, 0.5 to 8 μg/ml) were examined for their mechanisms of resistance. Five strains were typical of heterogeneous *S. aureus*: they gave positive reactions with a DNA probe specific for *mec* and contained a small fraction (10^{-6}) of highly resistant cells (MIC, >100 μg/ml). The rest of the 12 strains were homogeneous with respect to their methicillin resistance: the MIC of methicillin for all cells was 2 to 4 μg/ml, and no cells for which MICs were 50 μg/ml or higher were detectable ($<10^{-9}$). None of these strains reacted with the *mec*-specific DNA probe. One representative strain of each group was characterized in more detail. Strain CDC-1, prototype of heterogeneous methicillin-resistant *S. aureus*, contained penicillin-binding protein (PBP) 2a; its DNA could transform a methicillin-susceptible and novobiocin-resistant recipient to methicillin resistance with ca. 35% linkage to Nov^r. Introduction of the "factor X" determinant (K. Murakami and A. Tomasz, J. Bacteriol. 171:874–879, 1989) converted strain CDC-1 to high, homogeneous resistance. Strain CDC-6, prototype of the second group of isolates, showed completely homogeneous MICs of methicillin, oxacillin, and cefotaxime. The strain contained modified "normal" PBPs: PBPs 1 and 2 showed low drug reactivity (and/or cellular amounts), and PBP 4 was present in elevated amounts. No PBP 2a could be detected. DNA isolated from strain CDC-6 could transform the methicillin-susceptible and novobiocin-resistant strain to methicillin resistance in a multistep fashion, but this resistance showed no genetic linkage to the Nov^r marker. We suggest that staphylococci with borderline resistance may contain at least three different classes of mechanism: heterogeneous, methicillin-resistant *S. aureus*, PBPs of modified drug reactivities, and the previously reported hyperproduction of β-lactamase (L. K. McDougal and C. Thornsberry, J. Clin. Microbiol. 23:832–839, 1986).

Intrinsically resistant (methicillin-resistant) clinical strains of *Staphylococcus aureus* (MRSA) contain a supernumerary penicillin-binding protein (PBP), PBP 2a or 2′ (3, 4, 16). The genetic determinant of this 78-kilodalton protein has been cloned in *Escherichia coli* (1, 6, 9), and appropriate DNA probes constructed from the cloned DNA elicited positive reactions with all MRSA isolates tested so far while susceptible strains did not react, suggesting that the genetic determinant of PBP 2a may have originated in a species other than *S. aureus*, presumably as part of a transposable element (15). It is commonly assumed that the extremely high MIC for MRSA strains is related to the low-drug-affinity PBP 2a, which in the presence of antibiotics is supposed to take over the catalytic function(s) of normal PBPs in the synthesis of cell wall.

While PBP 2a or its genetic determinant or both have so far always been detected in MRSA isolates, a quantitative relationship between the cellular amounts of PBP 2a and the methicillin MIC for a particular strain could not be established: strains with high, homogeneous expression of resistance (MIC of methicillin, ≥800 μg/ml) contained the same amounts of PBP 2a as isolates in which 99.99% of the cells had MICs of 2 to 4 μg/ml (11). Recent analysis of the latter strains with "borderline" resistance to methicillin has led to the proposition that overproduction of β-lactamase may be one contributor to the antibiotic resistance of such isolates (10). We now report that, within a group of clinical isolates with a similar range of MICs (methicillin, 2 to 4 μg/ml; oxacillin, 2 to 8 μg/ml), it is possible to identify a new resistance mechanism that is independent of PBP 2a and β-lactamase production and seems to involve some modification of normal PBPs in a manner reminiscent of the mechanism of penicillin resistance in pneumococci (17) and of laboratory-isolated step mutants of *S. aureus* (14). We propose that these strains be referred to as MOD-SA (for "modified" PBP) in reference to their proposed resistance mechanism and also to distinguish them from the term BOR-SA, frequently used in the literature in reference to strains with borderline resistance, as defined by the particular nonmechanistic criteria of clinical microbiologists (10).

The sample of strains we examined was not meant to carry epidemiological significance, and the true incidence of

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TABLE 1. Some properties of the *S. aureus* strains used

Strain	Origin	Reference	β-Lactamase[a]	MIC (μg/ml)[b]			No. of highly resistant cells (methicillin MIC, ≥100 μg/ml) per 10^9 bacteria[c]	PBP 2a DNA probe	Tentative assignment of resistance mechanism[d]
				Methicillin[e]	Oxacillin	Cefotaxime			
COL	Highly resistant, homogeneous MRSA	1	−	800	800	>500	10^9	+	Homogeneous
RUSA4	Tn*551* mutant of COL	2	−	4	1	4	0	+	Homogeneous
RN2677	Methicillin susceptible, novobiocin resistant, rifamycin resistant	2	−	<0.8	0.2	1	0	−	Susceptible
CDC-1	Centers for Disease Control		−	4	1	8	20	+	Heterogeneous
CDC-2			+	4	8	16	200	+	Heterogeneous
CDC-3			+	4	4	≥16	200	+	Heterogeneous
CDC-4			+	2	1	16	150	+	Heterogeneous
CDC-5			+	4	8	16	150	+	Heterogeneous
CDC-6	Centers for Disease Control		−	4	4	16	0	−	MOD
CDC-7			+	4	1	8	0	−	MOD
CDC-8			+	4	1	4	0	−	MOD
S1	Lausanne, Switzerland		+	4	1	8	0	−	MOD
S2			+	4	2	8	0	−	MOD
S3			+	4	2	8	0	−	MOD
S4			+	4	1	8	0	−	MOD
S5			+	4	1	8	0	−	MOD
S6			+	4	1	8	0	−	MOD
NY1300	New York Hospital		ND	4	2	8	0	−	MOD
CHI82			ND	4	2	8	0	−	MOD
CHI83			ND	4	1	8	0	−	MOD

[a] β-Lactamase was determined by iodometric assay. ND, Not determined.
[b] Inoculum size was 10^4 CFU/ml.
[c] Tryptic soy agar containing 4 μg of clavulanic acid and 100 μg of methicillin per ml. Plates were evaluated after incubation at 37°C for 3 days. No significant increase in MICs was noted when the plates were incubated at 30 instead of 37°C.
[d] MOD, Modified PBP.
[e] Methicillin MICs in the presence or absence of clavulanic acid (4 μg/ml) showed no significant differences.

MOD-SA strains among clinical isolates remains to be determined.

MATERIALS AND METHODS

S. aureus clinical isolates with borderline levels of resistance to methicillin were obtained from several sources, and the relevant properties of these strains are summarized in Table 1. Tryptic soy broth and tryptic soy agar (Difco Laboratories, Detroit, Mich.) were used as growth media throughout. Bacterial stocks were kept frozen at −70°C in 10% sterile glycerol-containing medium. Overnight cultures were grown by diluting 100 μl (about 10^8 CFU) of bacterial stocks into 10 ml of tryptic soy broth and incubating at 37°C in glass tubes with aeration overnight (about 12 to 16 h). Overnight cultures were diluted into fresh, prewarmed tryptic soy broth (100-μl overnight culture to 10 ml of tryptic soy broth), and such cultures, in the middle of exponential growth phase, were then used in all physiological experiments. MICs were determined by agar dilution, using 10^3 to 10^4 CFU as inocula delivered onto the antibiotic-containing agar plates with a Steers replicator. Plates were evaluated after 2 and 3 days of incubation at 37°C. Population analysis was performed by plating various concentrations of bacteria on tryptic soy agar containing different concentrations of the antibiotics, and plates were incubated at either 37 or 30°C for 2 to 3 days. Penicillinase production was assayed by the iodometric method (12).

Preparation of membranes, assay of PBPs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography (4), and genetic transformation of various genetic markers (11) were done by the published procedures cited.

The DNA probe used for detection of the PBP 2a gene was obtained from John Kornblum, Public Health Research Institute, New York, N.Y. The origin of this probe was as follows (J. Kornblum, personal communication): the *mec* marker of the clinical MRSA strain COL was transformed into a methicillin-susceptible recipient strain; the 3.5-kilobase *Bgl*II fragment of the chromosomal DNA of a transformant was cloned into *E. coli* plasmid vector pUC8, following the procedures of Berger-Bachi and colleagues (2), and the 1.3-kilobase *Pst*I fragment was then subcloned into a p-Bluescript ks vector (Strategene, La Jolla, Calif.) and grown in *E. coli* JM109. This 1.3-kilobase fragment was found to be a specific probe for MRSA strains (D. Galetto, J. Froggatt, J. Kornblum, and G. Archer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, A59, p. 10).

Large-scale plasmid preparation from *E. coli* was done by standard methods (8), with the following modifications: phenol treatment followed by ether extraction was performed prior to cesium chloride-ethidium bromide centrifugation. After removal of the ethidium bromide from the plasmid band, the DNA was precipitated with ethanol, suspended in TE buffer (0.1 M Tris hydrochloride buffer, pH 7.5, containing 100 mM EDTA), and then extensively dialyzed against 6 liters of TE (pH 7.0).

S. aureus chromosomal DNA was prepared by the following modifications of the methods described by Lindberg et al. (7) and Berger-Bachi (2). Cells from 1-liter overnight

cultures in tryptic soy broth were washed and suspended in 100 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–100 mM EDTA. The cells were lysed for 1 h at 37°C with lysostaphin (70 μg/ml) and M1-muramidase (200 μg/ml). After pronase and sodium dodecyl sulfate treatment (7), the DNA was treated by the method of Berger-Bachi (2). After dissolving the DNA in TE buffer, extensive dialysis (as described above) of the plasmid DNA preparation was carried out. The DNA preparations were pure enough for restriction endonuclease digestion, and no degradation was observed.

Probing of the strains listed in Table 1 was performed in three ways. We performed dot blot hybridization of (i) crude lysates or (ii) purified DNA, using a Bio-Dot (Bio-Rad Laboratories, Richmond, Calif.) apparatus and following the manufacturer's instructions. (iii) In some experiments, chromosomal DNA preparations were treated with the restriction endonuclease *Eco*RI. Transfer of restricted chromosomal DNAs was performed by vacuum blotting (LKB Instruments, Inc., Rockville, Md.). Prehybridization and hybridization were done by standard methods (8) adapted to the use of GeneScreen membranes. Radioactive probe was prepared by using the Multiprime DNA-labeling system (Amersham Corp., Arlington Heights, Ill.).

RESULTS

The group of 17 *S. aureus* strains with borderline-level methicillin resistance (MICs, 2 to 4 μg/ml) could be divided into two classes (Table 1): strains CDC-1 through -5 each gave a positive reaction with the probe for *mec* DNA, and cultures of each one of these strains contained some highly methicillin-resistant cells (MIC, ≥100 μg/ml) at detectable, although very low, frequencies. The second class of resistant isolates did not react with the DNA probe, and cultures of these strains appeared to be homogeneous with respect to the methicillin MIC for all cells present. To facilitate subsequent PBP analysis, two β-lactamase-negative strains, CDC-1 and CDC-6, were selected as prototypes of resistance classes 1 and 2, respectively, and these two strains were analyzed in more detail for their mechanisms of resistance.

Properties of CDC-1. (i) Heterogeneous expression of methicillin resistance. The majority of cells in cultures of CDC-1 had a methicillin MIC of about 4 μg/ml. However, population analysis showed that such cultures also contained, at low frequencies, one or two subpopulations of highly resistant bacteria. Of 10^9 CFU plated, about 10^3 cells could grow up to 30 to 50 μg of methicillin per ml, and the MIC for about 10 to 20 cells was about 250 μg/ml (Fig. 1). An identical population analysis curve was obtained when the plates were incubated at 30 instead of 37°C (not shown).

(ii) Genetic transformation of resistance. Chromosomal DNA isolated from CDC-1 was used to transform competent cells of the methicillin-susceptible and novobiocin-resistant recipient strain RN2677. Strain CDC-1 was novobiocin susceptible. Transformants were selected on 0.3 μg of oxacillin per ml or 2 μg of methicillin per ml. The transformation frequency was 8×10^{-8}. Figure 1 shows that the heterogeneous methicillin resistance of the DNA donor CDC-1 was transformed into RN2677 (see population analysis curves for transformant t-28). Eight transformants were picked, purified, and tested for novobiocin resistance: three of eight transformants were also novobiocin susceptible, showing about 38% linkage between methicillin resistance and the novobiocin resistance locus.

Methicillin resistance from CDC-1 was also transformed into strain RUSA4, a transposon mutant of the highly and homogeneously resistant strain COL in which Tn*551* was inserted near or inside the PBP 2a gene, leading to the inactivation of PBP 2a production and a loss of methicillin resistance (MIC down from 500 to 800 to about 4 μg/ml). A unique property of RUSA4 is that, as a recipient in genetic transformations, it allows high and homogeneous expression of methicillin resistance even when the donor DNA is prepared from heterogeneously resistant strains (11).

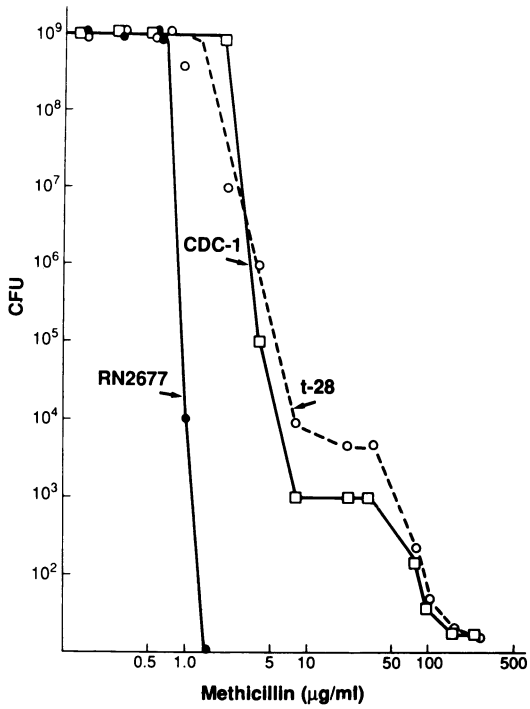

FIG. 1. Population analysis of the hetero-MRSA strain CDC-1 and its methicillin-resistant transformant t-28. Chromosomal DNA prepared from strain CDC-1 was crossed into RN2677; a methicillin-resistant transformant (t-28) was selected, and population analysis was performed, as described in Materials and Methods.

Transformants were selected on 4 or 8 μg of methicillin per ml, and the frequency of transformation was about 10^{-7}. Thirteen transformants were picked and purified; all were found to have greatly increased and nearly homogeneous expression of resistance (see population analysis curve of transformants t-42 and t-55 in Fig. 2). As expected, none of the transformants retained the erythromycin resistance of the recipient strain (11).

In a reciprocal cross, DNA from strain RUSA4 was used to transform the erythromycin resistance marker (Tn*551*) into strain CDC-1. Transformants were selected at 50 μg of erythromycin per ml. Such transformants no longer contained the highly methicillin-resistant subpopulations, and no PBP 2a could be detected in membranes prepared from an erythromycin-resistant transformant (not shown).

(iii) Production of PBP 2a. Membranes prepared from CDC-1 and from transformants t-28 and t-25 (constructed by transforming the methicillin resistance determinant of CDC-1 into RN2677) showed the presence of the 78-kilodalton PBP 2a in amounts comparable to that seen in the highly and homogeneously resistant strain COL (Fig. 3). Both the DNA donor strain CDC-1 and its methicillin-resistant transformant derivative t-42 (see titration in Fig. 4) appeared to have PBPs 1, 2, and 3 of normal antibiotic reactivity, and PBP 2a of

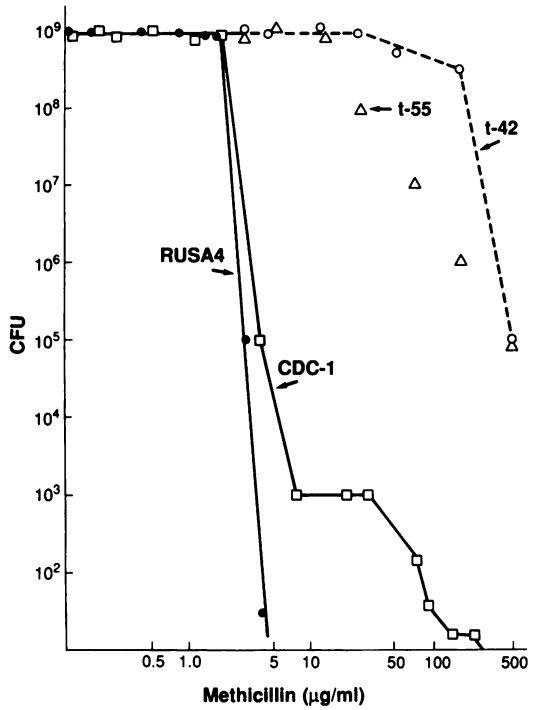

FIG. 2. Homogeneous expression of high-level methicillin resistance in transformants of CDC-1 introduced into the background of the Tn*551* mutant RUSA4. A competent culture of strain RUSA4 was used as recipient of donor DNA prepared from CDC-1.

CDC-1 showed the same low affinity for penicillin as that of strain COL (Fig. 4).

Properties of CDC-6. (i) Homogeneous expression of methicillin resistance. In contrast to CDC-1, cultures of strain CDC-6 did not contain highly resistant subpopulations of cells, but all bacteria present seemed to share common beta-lactam antibiotic MICs (about 4 μg/ml for methicillin and 16 μg/ml for cefotaxime) (Fig. 5).

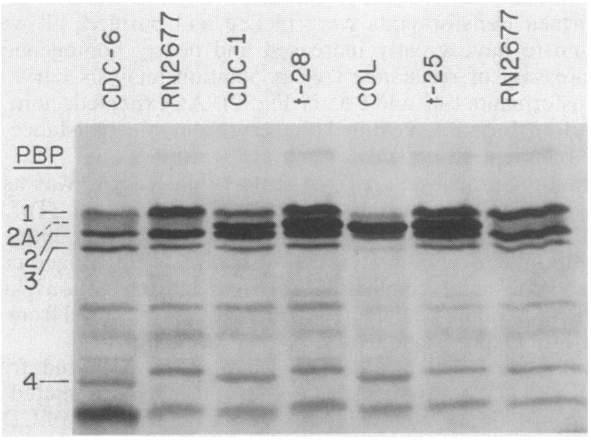

FIG. 3. PBP profiles of clinical strains CDC-1 and CDC-6, their genetic transformants, and some control strains. Membranes were prepared and assayed for PBPs as described in Materials and Methods. Strain COL is a highly resistant homogeneous clinical isolate; RN2677 is a methicillin-susceptible strain used as recipient in the crosses that yielded transformants t-25 and t-28.

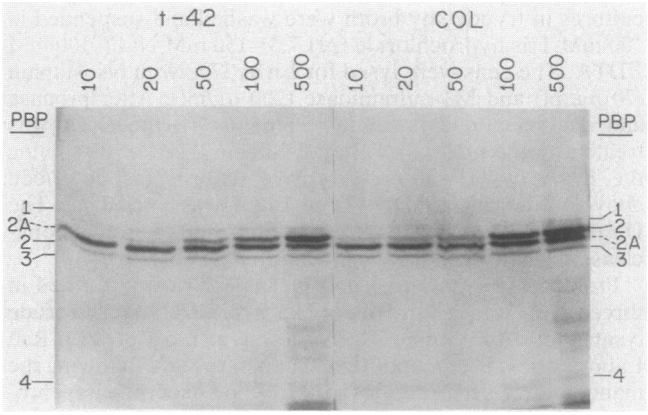

FIG. 4. Titration of the antibiotic activities of PBPs in strains COL and t-42. Membranes were exposed to [^{3}H]penicillin at the concentrations (nanograms per milliliter) indicated at the top of the lanes.

(ii) Genetic transformation. Chromosomal DNA isolated from CDC-6 could transform low-level methicillin resistance into the methicillin-susceptible and novobiocin-resistant RN2677. Selection was on 2 μg of methicillin per ml, 0.4 μg

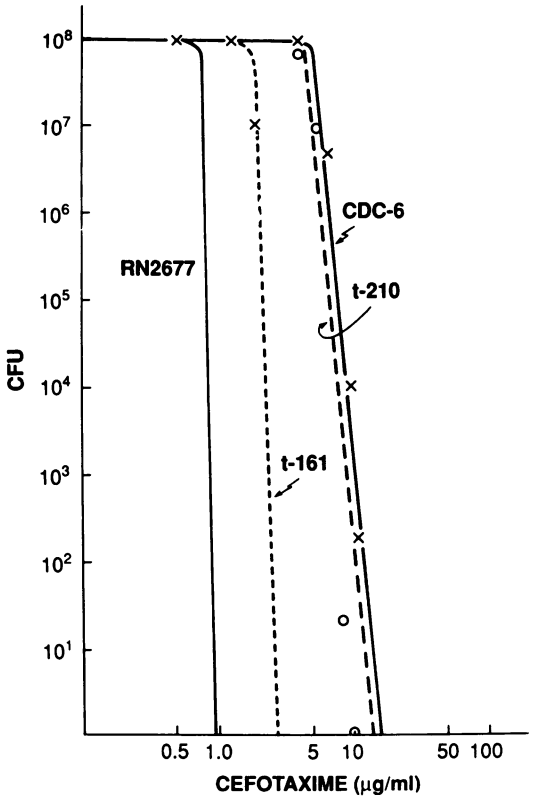

FIG. 5. Population analysis of the MOD-SA strain CDC-6 and its methicillin-resistant transformants. Chromosomal DNA prepared from CDC-6 was crossed into the methicillin-susceptible strain RN2677 and transformant t-161 was isolated, as described in the text. Next, a culture of t-161 was made competent and used as recipient for the next cross with donor DNA from CDC-6, yielding transformants with a further increased cefotaxime MIC (t-210). Population analysis was performed as described in Materials and Methods.

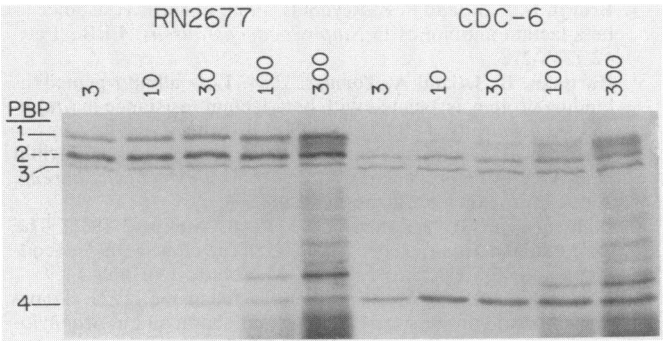

FIG. 6. Titration of the antibiotic reactivities of PBPs in the methicillin-susceptible control strain RN2677 and strain CDC-6.

of oxacillin per ml, or 12 μg of cefotaxime per ml; transformation frequencies were 10^{-8} to 10^{-9}. Because of the more convenient range of MICs, cefotaxime was used for analysis of transformants. A total of 24 transformant colonies were analyzed further by replica plating. All transformants were able to grow on increased levels of methicillin (2 μg/ml), oxacillin (0.4 μg/ml), or cefotaxime (10 μg/ml) (Fig. 5). In contrast to transformants of CDC-1, all 24 of the CDC-6 transformants retained the novobiocin resistance of the recipient strain. Also in contrast to the similar genetic cross with CDC-1, using RUSA4 as a recipient and CDC-6 DNA as the donor did not result in transformants with increased levels of methicillin resistance.

(iii) PBPs. Membranes prepared from CDC-6 showed no detectable PBP 2a (Fig. 3). CDC-6 membranes and membranes from the methicillin-susceptible strain RN2677 at identical protein concentrations were exposed to different concentrations of radioactive penicillin. In CDC-6, PBPs 1 and 2 showed markedly lower labeling, while a PBP with the electrophoretic mobility of PBP 4 had greatly increased labeling, as compared with appropriate PBPs in the susceptible strain (Fig. 6). In the genetic transformant t-161 (see Fig. 5 for population analysis), the decreased labeling of PBP 1 was also transformed along with increased resistance to methicillin. However, decreased labeling of PBP 2 and the increase in drug binding to PBP 4 were not seen in this transformant (Fig. 7).

DISCUSSION

The findings described here show that *S. aureus* isolates with beta-lactam MICs characteristic of strains with low to borderline resistance may include at least two mechanistically distinct classes. The first one of these, CDC-1 (representing, in our sample, five of the 17 isolates examined), contained strains with typical features of heterogeneous MRSA: i.e., positive reaction with the *mec*-specific DNA probe; detectable levels of PBP 2a, and presence of the *mec* determinant (identified by genetic transformation) linked to the Nov^r locus. Also characteristic of heterogeneous MRSA was the dramatically increased MIC in transformants obtained by crossing the DNA of these strains into the Tn*551* transposon mutant RUSA4. It has been shown that, in the genetic background of RUSA4, the PBP 2a genes of several heterogeneous MRSA DNA donors have acquired homogeneous expression of high-level resistance (11). All methicillin-resistant transformants of CDC-1, in both the RN2677 and the RUSA4 backgrounds, produced PBP 2a. Perhaps less typical for heterogeneous strains was the extremely low frequency of the highly resistant subpopulation (in the order of 10^{-7}).

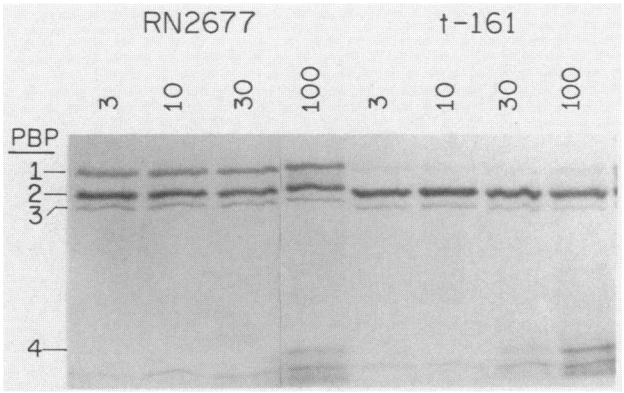

FIG. 7. Titration of the antibiotic reactivities of PBPs in transformant t-161.

The majority of strains (11 of 17) examined in our study belonged to a second class of resistance mechanism and showed contrasting features with the representative of the heterogeneous MRSA class in each of the tests performed. Isolates belonging to this second class did not react with the *mec*-specific DNA probe; cultures of these strains contained no detectable numbers of highly resistant cells (frequency, $<10^{-9}$); and the representative strain, CDC-6, contained no detectable PBP 2a. While genetic transformation of the methicillin resistance of CDC-6 was possible, transformants showed no linkage to the novobiocin resistance determinants. Transformation of methicillin resistance into the transposon mutant RUSA4 gave no increased resistance levels over that of the DNA donor, and the transformants retained the Tn*551* marker (erythromycin resistance). These results indicate that the methicillin resistance marker(s) in CDC-6 occupied a chromosomal position different from that of the *mec* marker of typical MRSA strains.

Our results support the conclusions reached by Chambers et al. and Sierra-Madero et al. (H. Chambers, G. Archer, M. Matsuhashi, M. Sachdeva, and S. Kennedy, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 247, 1988; J. Sierra-Madero, B. Yen-Lieberman, C. C. Knapp, and J. A. Washington, 28th ICAAC, abstr. no. 248, 1988), who have independently suggested similar mechanism of resistance in some low- or borderline-level resistant strains.

The outstanding new feature of CDC-6 was the presence of PBPs 1, 2, and 4 with modified penicillin-binding capacities: PBPs 1 and 2 appeared to have strikingly lower penicillin-binding capacities than the same proteins in the susceptible strain RN2677. A protein with the electrophoretic mobility of PBP 4 appeared to bind penicillin more avidly in CDC-6 than in RN2677. In the genetic transformant t-161, decreased penicillin binding by PBP 1 accompanied the increased antibiotic resistance. The lack of decreased binding by PBP 2 and the lack of increased binding by PBP 4 in t-161 may be related to the fact that t-161 does not yet have the full resistance level of the DNA donor CDC-6 (Fig. 5).

While our studies clearly identify abnormality of antibiotic binding by PBPs 1, 2, and 4, they are not appropriate to distinguish between changes in kinetic properties, per-cell concentration, deacylation rates, etc., of these PBPs, i.e., features that may contribute to the observed alterations. The properties of CDC-6 show close similarities to the PBP-linked, beta-lactam-resistant mutants of pneumococci, gonococci, and some other pathogens (13). We suggest, provi-

sionally, that these mechanistically new mutants of staphylococci with modified PBP reactivities be referred to as MOD strains. In the evolutionary context, the existence of MOD-type resistance mutants of staphylococci should have been predictable: we propose that such strains have been selected by direct antibiotic pressure in a manner that has been shown to occur during exposure of susceptible staphylococci to increasing concentrations of beta-lactam antibiotics in the laboratory (14). It is most likely that selection for this type of resistance mechanism continues now, and it is also likely that the specific types of PBP alterations associated with increased MICs would reflect the specific beta-lactam antibiotic used in the particular clinical environment, as was shown in mutants selected in the laboratory (14). The appearance in various parts of the world of MRSA strains equipped with the PBP 2a gene probably reflects the spread of a single successful resistance mechanism in combination with various auxiliary mutations that seem to regulate the degree of resistance (MIC) that the acquisition of the PBP 2a gene can provide (11, 13).

While the actual MIC increase associated with the MOD type of resistance is modest by comparison with those linked to the PBP 2a type of resistance, the discovery of MOD staphylococci indicates the emergence of a new resistance mechanism among clinical strains that, eventually, may lead to substantially higher MICs with potential clinical implications. In the laboratory, it has been possible to select *S. aureus* mutants for which methicillin MICs were >100 μg/ml (14).

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